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(57) Abstract

Cells of the clones CN21 and DB30S and DB40 express a form of an acetylcholine receptor (AChR) containing an adult ϵ subunit. The AChR is useful in an assay for AChR antibodies which is diagnostic of myasthenia gravis.

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CELL LINES EXPRESSING AN ACETYLCHOLINE RECEPTOR, CONTAINING AN EPSILON SUBUNIT

INTRODUCTION

Muscle-type nicotinic acetylcholine receptors (AChR) are composed of four homologous subunits. The subunits assemble into a pentamer around a central transmembrane, ACh-gated cation channel. In mammalian muscle there are two subtypes of the AChR, the extrajunctional or fetal subtype composed of α_2, β, γ δ subunits and the junctional or adult subtype in which an ε subunit replaces the γ subunit within the AChR pentamer [1]. Although the adult and fetal AChR subtypes both bind to the snake neurotoxin α-bungarotoxin (α-BuTx) with high affinity they have different electrophysiological, immunological and biochemical properties [reviewed in ref.2].

Myasthenia gravis (MG) is an autoimmune disease mediated by antibodies directed against muscle AChR. These antibodies show high specificity for the native human AChR. Thus, for diagnostic and other studies in MG it is necessary to have a source of human muscle AChR. Two common sources are amputated leg muscle, which is both difficult to obtain and contains only small amounts of adult AChR, and the rhabdomyosarcoma cell line TE671 (USP 4789640).

AChRs derived from the TE671 cell line have been extensively characterised [3,4,5,6]. In these cells the fetal AChR subtype is expressed on the cell surface, but in detergent extracts up to 50% of ^{125}I-α-BuTx binding is found on unassembled or partially assembled α subunits that are not inserted into the surface membrane [5,7]. In common with many other cultured muscle cell lines, TE671 cells express levels of the AChR ε subunit that are not detectable in radioimmunoassays.

INVENTION

We have transfected the TE671 cell line with cDNA encoding the human muscle AChR ε subunit [8] and established a stable clonal cell line which constitutively expresses the human AChR adult subtype at levels 2- to 3-fold higher than the endogenous fetal subtype. The presence of high levels of junctional AChR in the cell extracts will improve the use of TE671 cells as the source of AChR in the diagnostic radioimmunoassay for MG [9], and the presence of functional adult AChR on this cell line will make it useful for pharmacological studies on the human AChR subtype that is responsible for signal transmission at the adult endplate.

A sample of our cell line CN21 has been deposited at the European Collection of Animal Cell Cultures at Porton Down under Budapest Treaty conditions and has received an accession number N21-95092116 and a deposit date of 21 September 1995..

The CN21 cell line has been modified to generate higher levels of expression of the adult from of AChR by stably transfecting the CN21 lines with expression vectors containing cDNA encoding the human muscle AChR β subunit [24] and the human muscle AChR δ subunit [6; 8].

Samples of our cell lines DB30S and DB40 have been deposited at the European Collection of Animal Cell Cultures at Porton Down under Budapest Treaty conditions and have received accession numbers and deposit dates as follows:

DB30S	96110510	5 November 1996
DB40	96110509	5 November 1996

The invention provides cells of the clones CN21 and DB30S and DB40 of the line TE671, and mutants thereof which express the acetylcholine receptor ε subunit.

The invention also provides acetylcholine receptors and subunits thereof which are expressed by the cells of these clones. These may be labelled with a signal moiety whose nature is not material to the

invention but which may conveniently be ^{125}I-labelled α-bungarotoxin.

The invention also includes use of these acetylcholine receptors and subunits thereof in *in vitro* biochemical assays for myasthenia gravis. The assay may involve incubating the labelled acetylcholine receptor with a sample of patient serum suspected of containing antibodies associated with myasthenia gravis, and determining the amount of the signal moiety associated with the antibodies.

Local anaesthetics act on AChRs at the neuromuscular junction. They have been shown to block the increase in ionic conductance in response to agonist binding to AChR and in addition may increase the rate of AChR desensitisation. It has been shown that fetal AChR subtype is less sensitive to d-tubocurarine than the adult AChR subtype [22] with respective apparent dissociation constants of 5.5×10^{-7} M and 4.5×10^{-8} M [23]. The cell lines described herein provide a new source of membrane bound adult AChR and will enable the contrasting action of drugs acting on fetal and adult AChR subtypes to be effectively measured.

Reference is directed to the accompanying drawings in which:-

Figure 1 is a bar chart showing anti-AChR concentrations in different patients sera determined by different methods; and

Figure 2 is a bar chart comparing α-bungarotoxin binding in different transfected cell lines.

EXAMPLE 1

MATERIALS AND METHODS

Expression Plasmid

phεNA was constructed by blunt end ligation of cDNA encoding the human muscle AChR ε subunit [8] into the *Eco*R V site of pcDNA3 (Invitrogen) and the ε subunit cDNA orientation within the plasmid was established by diagnostic restriction endonuclease digestion. The

plasmid contains the neomycin resistance gene for selection of G418-resistant stable cell lines, and constitutive expression of the cDNA insert is driven by the cytomegalovirus promoter.

Cell culture and transfection

TE671 cells and transfected cell lines and clones were grown under conventional conditions as previously described [6]. 5μg of pheNA was used to transfect 2 x 10^5 cells in 6-well 60 mm tissue culture dishes. Transfections were carried out using Transfectam reagent (Promega) according to the manufacturer's instructions. Cells were allowed to recover for 48 hours after transfection before selection for 1 week in medium containing 1 mg/ml geneticin (Gibco-BRL) and then for 2 weeks in medium containing 2 mg/ml geneticin. Resistant cells were cloned by limiting dilution (0.1 cell/well, 3 x 48-well tissue culture plates) and the clones subsequently maintained with 0.5 mg/ml geneticin added to the medium.

RESULTS

Transfection of TE671 cells

TE671 cells express an AChR composed of α, ß, γ and δ subunits. TE671 cells were transfected with cDNA encoding the human muscle ε subunit. Following selection for stable transfectants cells were cloned by limiting dilution. Ten clonal cell lines were grown up and assayed with the AChR γ- and ε-subunit-specific rabbit antisera. The specificity of the antisera was first confirmed by immunoprecipitation assays on either fetal or adult human AChR expressed in *Xenopus* oocytes [15]. Total ^{125}I-α-BuTx binding was determined using the rabbit polyclonal serum raised against a region within the cytoplasmic domain of the α subunit [16]. Initially, three of the ten clones (CN10, CN21 and CN33) gave ^{125}I-α-BuTx binding following immunoprecipitation with the ε-subunit-specific antisera. In each of these three clones the ^{125}I-α-BuTx binding

precipitated by the γ-subunit-specific antisera was reduced when compared to the remaining seven clones. Continuous culture of the clones resulted in the loss of the ε-subunit expression from clones CN10 and CN33; however clone CN21 has maintained expression of the AChR ε subunit in culture for over nine months.

RNase protection assays

The level of mRNA encoding the ε and γ AChR subunits in clones CN21 and GN46 was assessed by RNase protection assays. Clone GN46, which was resistant to G418 selection but did not express the ε subunit was used as a control. When an antisense ε cRNA probe was used, clone CN21 gave a 175 bp protected fragment indicating expression of mRNa encoding the AChR ε subunit, whereas there was no protected fragment for clone GN46. When an antisense γ-subunit cRNA probe was used both clone CN21 and clone GN46 gave a 207 bp protected fragment, indicating that mRNA encoding the AChR γ subunit is expressed at a similar level in the two clones. Although precise quantitative assays have not been performed, densitometric scans of the RNase protection assays indicate that there is approximately five times as much mRNA encoding the ε subunit as mRNA encoding the γ subunit in clone CN21 cells.

Assay for expression of AChR γ and ε subunits

The comparative level of expression of the γ and ε subunits within the CN21 clonal cell line was determined by immunoprecipitation of ^{125}I-α-BuTx binding sites. The level of expression of AChR in the control cell line GN46 is similar to untransfected TE671 cells. The γ-subunit-specific antisera precipitated 60% of the maximum ^{125}I-α-BuTx from GN46 cells and 22% from the CN21 cells. The ε-subunit-specific antisera gave background binding with GN46 cells and precipitated 53% of the maximum ^{125}I-α-BuTx from the CN21 clone. CN21 and GN46 cells, grown on 60 mm

6-well tissue culture plates, were assayed for total and cell surface ^{125}I-α-BuTx binding sites, and the extracted AChRs immunoprecipitated with mab F8, which is specific for the AChR fetal subtype, and G10, which precipitates both forms [11]. In contrast to the AChR subunit-specific rabbit antisera, both these mabs recognise the AChR extracellular domain. CN21 cells, expressing the ε subunit, showed higher levels of ^{125}I-α-BuTx binding than GN46 cells or untransfected TE671 cells. The fetal-AChR-specific mab F8 precipitates 50% of the total ^{125}I-α-BuTx from GN46 cells but only about 25% of the total ^{125}I-α-BuTx from CN21 cells. It precipitated almost all surface ^{125}I-α-BuTx AChR from GN46 cells and only 25% from CN21 cells.

Electrophysiology

In order to establish that the ε subunit is incorporated into functional adult subtype AChR pentamers within the cell membrane, we carried out analysis of the ACh-activated single channel currents. Previous reports [1,13,14,15] have shown ε-AChR channels are characterised by a larger channel conductance and shorter mean open times than γ-AChR channels. The lower-conductance AChR is the endogenous channel of TE671 cells [4]. The distribution of current amplitudes of ACh-gated single channel currents in GN46 cells exhibited one peak of mean amplitude 4.5 pA, whereas in CN21 cells there were two well-separated peaks of 4.7 and 6.9 pA (at -120 mV). In both cell lines the ACh-gated single channel currrents reversed close to 0 m V and had linear current-voltage relationships. The slope conductances were 39.2 ± 2.3 pS (n=3 patches) for channels in GN46 and 39.8 ± 2.8pS and 60.2 ± 1.5 pS (n=4 patches) for the channels in CN21 cells. The properties of the two channels match those observed for calf, rat mouse and human adult and fetal AChRs expressed in *Xenopus* oocytes or COS cells [1,13,14,15]. The additional higher-conductance AChR present in the CN21 cell line shows properties

similar to the major AChR channel species at the normal human endplate [17].

EXAMPLE 2

Introduction:

Antibodies to AChR are diagnostic for myasthenia gravis. The antibodies bind to the adult AChR at the neuromuscular junction and lead to defects in neuromuscular transmission, with consequent muscle weakness. In embryonic or denervated muscle, a fetal-type AChR is produced which differs in one subunit from the adult form. Most MG sera bind equally well to both forms, or even better to the fetal form (which is present in the adult thymus). However, some patients with very low anti-AChR values bind better to the adult form [20]. In the immunoassay originally described for anti-AChR in MG [18;21], a mixture of adult and fetal-type AChR extracted from amputated leg muscle was used; however, the proportion and concentration of the adult-type AChR was always low because of the small amount present in adult muscle. Moreover, some laboratories have subsequently used AChR extracted from the TE671 cell line; this contains essentially only fetal-type AChR and leads to false negative results in a proportion of patients [19].

Methods:

Immunoprecipitation of ^{125}I-α-BuTx-AChR by myasthenia gravis (MG) sera

To see whether MG sera show higher titres with AChR extracted from the ε-transfected TE671 cells compared with the untransfected cells, 10 sera which had previously been shown to have "equivocal" titres (0.2-0.49nM; i.e. on the margin between normal values (<0.2nM) and clear positive values (>.49nM)) of anti-AChR antibody were tested. 5µl of each serum was incubated overnight with a 50 µl aliquot of

^{125}I-α-BuTx-labelled extract in a total volume of 100 µl. Complexes were precipitated by 50 µl of goat antiserum to human IgG. The precipitates were pelleted and washed [21]. Results were compared with those in serum from MG patients with undetectable anti-AChR antibodies, and with standard controls (one high positive, one low positive, two healthy controls). The ε-AChR preparations contained between 1 and 1.5 pmoles/ml of AChR. The commercial preparation of AChR (γ-AChR, RSR Ltd) from untransfected TE671 cells contained about 2 pmoles/ml. Cpm precipitated by healthy control sera were subtracted from test results, and immunoprecipitation expressed as nmoles of α-BuTx binding sites precipitated/litre of serum.

Results:

In the graph of Figure 1 the results are shown plotted against the titres (shown in figures below the X axis ranging from 0.2 nM to 0.4 nM) obtained in a routine assay which uses a mixture of extracts from amputated muscles, that include fetal type and a small amount of adult. Five of the ten, previously equivocal, sera showed higher reactivity with ε-AChR than with γ-AChR, placing the results in the positive range (>.5nM). Four showed similar results with each preparation, but better than those obtained in the routine assay. Only one serum showed better reactivity with γ-AChR than with ε-AChR. Overall, use of the preparation of ε-AChR would enable positive results to be reported on 8/10 of the sera that had previously been determined as equivocal. This would increase the number of positive results by about 7% and reduce the number of equivocal results accordingly.

None of the seronegative MG sera showed equivocal or positive titres against either of the two preparations (data not shown).

EXAMPLE 3

Construction of an expression vector for the AChR β subunit, phβZeo.

cDNA encoding the AChR β subunit [24] was ligated into the expression vector pZeoSV (Invitrogen) cut with *EcoR I/BCl* I to generate phβZeo. Expression of the AChR β subunit gene is under control of the SV40 enhancer/promoter, and the vector in addition gives resistance to the antibiotic zeocin.

Construction of an expression vector for the AChR δ subunit, phδPurcmv

A mammalian expression vector giving resistance to puramycin was constructed by ligating the *Steptomyces alboniger* puramycin-N-acetyl-transferase gene expression cassette into pcDNA3 cut with *Dra* III/*Bst*1107 I to generate an expression vector pPurcmv. Cells expressing puramycin-N-acetyl-transferase are resistant to the antibiotic puromycin. cDNA encoding the AChR δ subunit [8] was ligated in at the EcoRV site within the polylinker sequence of pPurcmv to generate a vector that will express the AChR δ subunit under transcriptional control of cytomegalovirus promoter, and give resistance to puramycin.

Cell culture and transfections

Cell culture, transfections and cloning were preformed essentially as described in Example 2. Line DB30S was established by transfection of cell line CN21 with phβzeo, selection of zeocin resistant clones, and the selection of clones that show increased ^{125}I-α-BuTx binding by radioimmunoprecipitation. Line DB40 was established by transfection of DB30S with phδPurcmv, establishing clones resistant to G418, zeocin and puramycin and selecting clones that show increased levels ^{125}I-α-BuTx binding as measured by radioimmunoprecipitation assay.

5×10^5 cells from each of the cell lines TE671, CN21, DB30S and DB40 were plated on 3 wells of a 6-well tissue culture plate. Cells were grown to confluence, AChR extracted in Triton X-100, and ^{125}I-α-BuTx binding determined by radioimmunoprecipitation assay. Results are expressed as cpm/well (mean of 3 wells). 200 cpm = 1 fmol.

Results

Results of assays in which levels of α-BuTx binding in cell lines TE671, CN21, DB30S and DB40 are shown in Figure 2. CN21, DB30S and DB40 all show higher levels of 1 November 1996-BuTx binding than in TE671 cells with DB40 showing an approximately eight fold increase. The higher levels of ^{125}I-α-BuTx binding obtained from these modifications of the CN21 cell line should additionally enhance their effectiveness for use in the diagnostic assay for myasthenia gravis.

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CLAIMS

1. Cells of the clone CN21 of the cell line TE671, and mutants thereof, which express the acetylcholine receptor ε subunit.

2. Cells of the clone DB30S of the cell line TE671, and mutants thereof which express the acetylcholine receptor ε subunit.

3. Cells of the clone DB40 of the cell line TE671, and mutants thereof which express the acetylcholine receptor ε subunit.

4. An acetylcholine receptor or subunit thereof which is expressed by the cells of any one of claims 1 to 3.

5. An acetylcholine receptor or subunit thereof as claimed in claim 4 which is labelled with a signal moiety.

6. An acetylcholine receptor or subunit thereof as claimed in claim 5, wherein the signal moiety is ^{125}I labelled α-bungarotoxin.

7. Use of the acetylcholine receptor of any one of claims 4 to 6 in an *in vitro* biochemical assay for myasthenia gravis.

8. In an assay for myasthenia gravis which comprise providing an acetylcholine receptor labelled with a signal moiety, incubating the labelled acetylcholine receptor with a sample of patient serum suspected of containing antibodies associated with myasthenia gravis, and determining the amount of the signal moiety associated with the antibodies, the improvement which comprises using the acetylcholine receptor derived from cells according to any one of claims 1 to 3.

9. Use of the CN21 or DB30S or DB40 cell line or preparations from these cells for the study, development and evaluation of chemicals and drugs acting at the neuromuscular junction.

Fig.1.

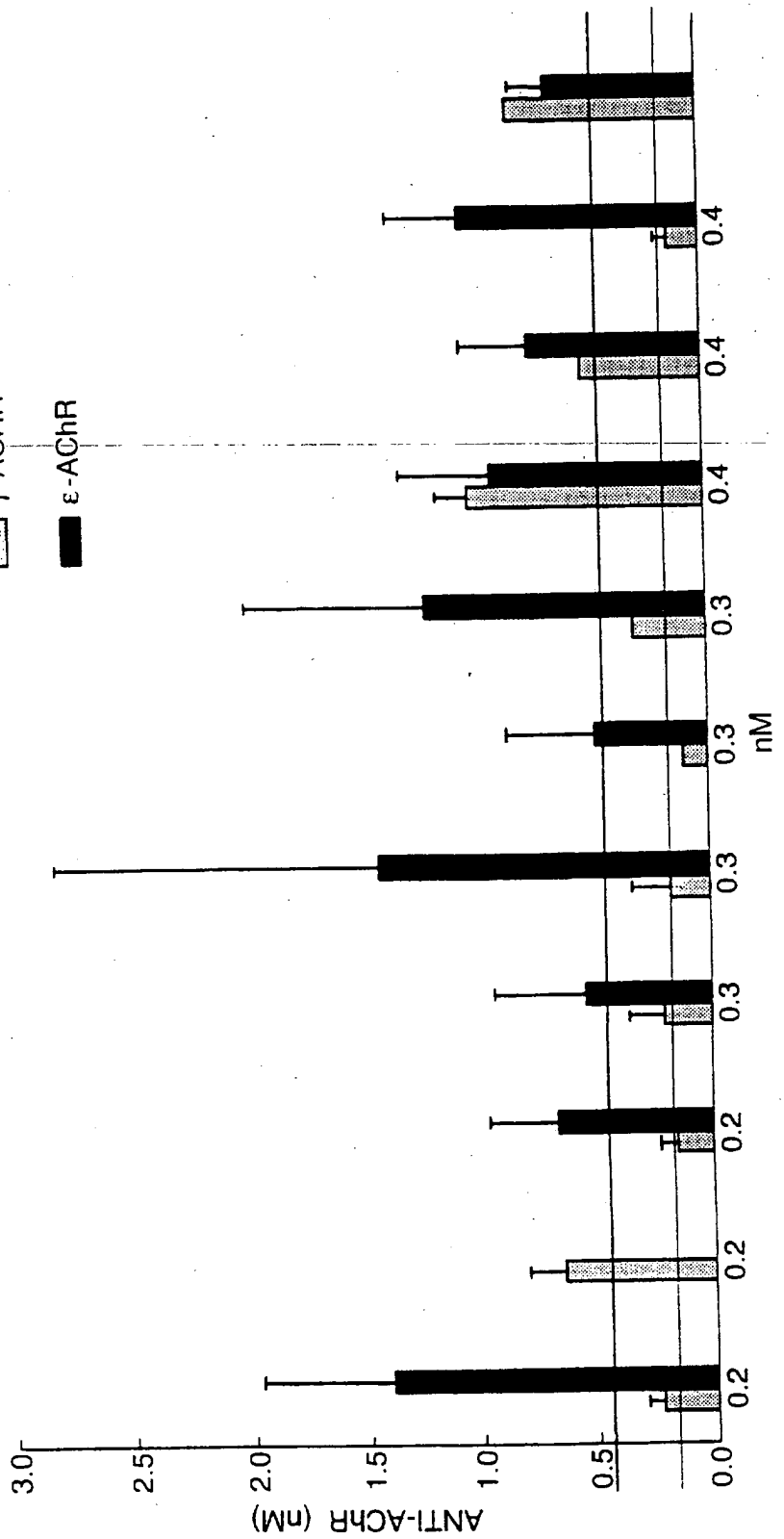
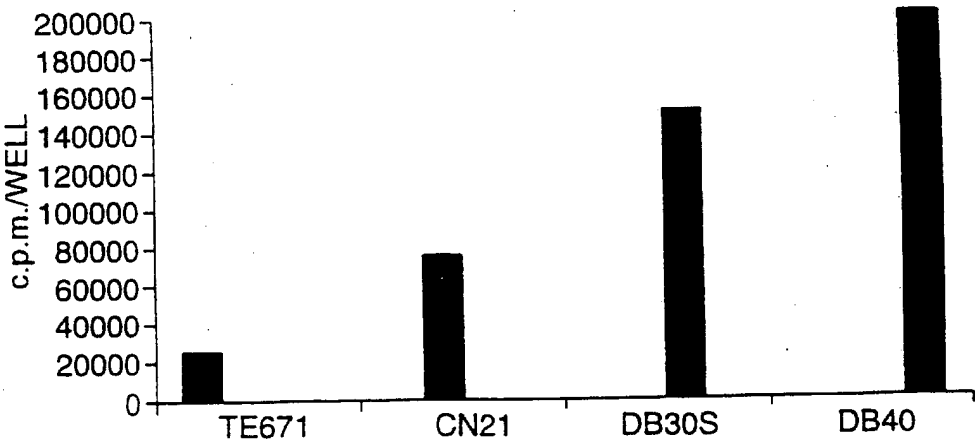


Fig.2.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TE671
CN21
DB30S
DB40

INTERNATIONAL SEARCH REPORT

Inte. onal Application No
PCT/GB 96/02725

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/10 C07K14/705 G01N33/564 G01N33/566

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF NEUROIMMUNOLOGY, vol. 51, no. 1, April 1994, pages 63-68, XP000615670 F.E.SOMNIER: "Anti-acetylcholine receptor (AChR) antibodies measurement in myasthenia gravis: The use of cell line TE671 as a source of AChR antigen" see the whole document ---	4-7
X	EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 215, no. 2, July 1993, pages 229-238, XP000615672 D.BEESON ET AL.: "Primary structure of the human muscle acetylcholine receptor. cDNA cloning of the gamma and epsilon subunits" see page 237 --- -/--	4

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

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3

Date of the actual completion of the international search	Date of mailing of the international search report
6 February 1997	14.02.97
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INTERNATIONAL SEARCH REPORT

Inter. nal Application No
PCT/GB 96/02725

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	NEUROSCIENCE LETTERS , vol. 207, no. 1, 22 March 1996, pages 57-60, XP000644311 D.BEESON ET AL.: "Stable functional expression of the adult subtype of human muscle acetylcholine receptor following transfection of the human rhabdomyosarcoma cell line TE671 with cDNA encoding the epsilon subunit" see the whole document ---	1,4-9

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